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**Region- and layer-specific activation of the higher order auditory cortex Te2 after remote retrieval of fear or appetitive memories.**

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*Running title: Neural networks of fear and appetitive memories in the auditory cortex*

**Abstract**

The auditory cortex is involved in encoding sounds which have acquired an emotional-motivational charge. However, the neural circuitry engaged by emotional memory processes in the auditory cortex is poorly understood. In this study, we investigated the layers and regions that are recruited in the higher order auditory cortex Te2 by a tone previously paired to either fear or appetitive stimuli in rats. By tracking the protein coded by the immediate early gene *zif268*, we found that fear memory retrieval engages layers II-III in most regions of Te2. These results were neither due to an enhanced fear state nor to fear-evoked motor responses, as they were absent in animals retrieving an olfactory fear memory. These layers were also activated by appetitive auditory memory retrieval. Strikingly, layer IV was recruited by fear, but not appetitive memories, whereas layer V activity was related to the behavioral responses displayed to the CS.

In addition to revealing the layers and regions which are recruited in the Te2 by either fear or appetitive remote memories, our study also shows that the neural circuitry within the Te2 that processes and stores emotional memories varies on the basis of the affective-motivational charge of tones.

**Keywords**: auditory cortex, emotional learning, expression of immediate early genes, long-term memory, cortical circuits

## Introduction

During emotionally-laden experiences, perceived stimuli acquire emotional-motivational charges that drive future choices and behaviors. This process has been referred as "emotional learning". From the seminal studies of Scheich and colleagues (Brosch et al. 2011; Ohl and Scheich 2005) and Weinberger and colleagues (Weinberger 2004, 2007, 2015), it is becoming clearer that sensory cortices, such as the auditory cortex, participate in the formation and maintenance of emotional memories, and that the role played by these structures in emotional memory goes beyond simple sensory stimuli perception and memorization (reviewed in Fritz et al. 2007; Grosso et al. 2015a; Shamma and Fritz 2014; Weinberger 2004, 2007, 2015). In support of this notion, we have identified higher order components of sensory cortices, such as the temporal auditory cortex Te2, which are essential for storing remote fearful memories (Cambiaghi et al. 2015; Grosso et al. 2015b; Sacco and Sacchetti 2010).

Intriguingly, many studies have shown that the learned emotional-motivational charge of sounds shapes the activity of neurons in the primary (Bieszczad and Weinberger 2012; David et al. 2012; Letzkus et al. 2011; Pi et al. 2013; Yin et al. 2014) and higher order (Bao et al. 2001; Carretta et al. 1999; Kown et al. 2012; Grosso et al. 2015b; Sacco and Sacchetti 2010) auditory cortex. For example, it has been shown that the emotional content provided by similar auditory stimuli, but paired to either an aversive or an appetitive stimulus, was able to shape the plasticity of the auditory cortical receptive field in opposite ways (David et al. 2012; Yin et al. 2014). Taken together, these findings suggest that associative and motivational processes are pronounced within the auditory cortex, where they can help to link the physical attributes of the stimuli to the affective charge or to the behavioral responses occurring during emotional experiences (Cambiaghi et al. 2016; Grosso et al. 2015a, 2015b). Nevertheless, a comprehensive picture of the circuits that are engaged by emotional associative processes within the auditory cortex of mammals is still lacking. Specifically, it is not yet understood whether sounds paired to positive or negative experiences are processed and

stored in different anatomical assemblies within the auditory cortex, or whether these emotional memories are encoded in largely overlapping cortical circuits. Furthermore, it is unknown whether the contrasting emotional memories recruits cortical layers I-VI in a similar way, or if there are layer-specific circuits within the auditory cortex for encoding positive or negative memories.

Therefore, in the present study we addressed two related questions. Firstly, we investigated the regions and the layers recruited in the Te2 during the retrieval of remote auditory fear memories. For this purpose, we tracked the activity-dependent induction of the immediate early gene (IEG) *zif268* (also known as EGR1) in Te2 following the retrieval of remote fear auditory memories. Among IEGs, the expression of zif268 has been associated with long-term plasticity occurring during learning and memory retrieval (Frankland et al. 2004; Xie et al. 2014). Accordingly, several recent studies have shown changes in zif268 expression in the sensory cortex following emotional memory processes (Hall et al. 2001; Kwon et al. 2012; Maviel et al. 2004; Sacco and Sacchetti 2010). Then, we investigated how Te2 activity was shaped by presenting identical tones that had been previously paired to appetitive stimuli. Taken together, these analyses are aimed at identifying the layers and regions engaged by either fear or appetitive memories and, at the same time, verifying whether these opposite emotional memories recruit different or overlapping circuits within the Te2 auditory cortex.

## Materials and Methods

**Subjects.** Male Wistar rats (age: 65-75 days, weight: 250-320 g, from Harlan Italy, Verona, Italy) were used in this study. Animals were housed in plastic cages with food and water available *ad libitum*, and subjected to a 12 h light/dark cycle (lights on at 7:00 A.M.) at a constant temperature of 22 ± 1°. All experiments were conducted in accordance with the European Community Council Directive 2010/63/EU and were approved by the Italian Ministry of Health (authorization no. 265/2011).

**Behavioral Procedures.**

**Fear conditioning.** For *fear conditioning training*, rats were gently placed in a basic Skinner box module (Rat Test Cage, Coulbourn Instruments, Allentown, PA, USA**)** provided with a loudspeaker to emit acoustic stimuli of known intensity, frequency and duration. Box dimensions were 30 x 28 x 35 cm. The top and two opposite sides were made of aluminum. The remaining two sides were made of transparent plastic. The floor was made of stainless steel rods connected to a shock delivery apparatus (Precision Animal Shocker, Coulbourn Instruments). This apparatus was enclosed within a sound attenuating chamber (Isolation Cubicle, Coulbourn Instruments). Once inside the apparatus, animals were left undisturbed for 2 min. After this time, a series of acoustic CSs (3000 Hz tone, amplified to 75 dB and lasting 8 s) were administered seven times, at 22 s intervals. The final 1 s of each CS was paired with a US consisting of an electric foot shock (intensity, 0.70 mA) (fear conditioned-tone). In the fear conditioned-odor group, seven almond odors (12 s, 48-s inter-trial interval) were presented using a flow-dilution olfactometer. Clean air (1.5 L/min) was directed to a solenoid valve that, when operated, transferred the air to a 15 ml bottle containing 10 ml of almond odor. Odorized air was then directed to the conditioning chamber via ¼ inch Tygon tubing.

*Remote fear memory retention* was tested at 1 month after conditioning. Rats were placed in a different apparatus from that used for conditioning, and in a different room, in order to avoid conditioned fear behavior related to contextual cues (Sacco and Sacchetti 2010). For this purpose, the apparatus was a transparent plastic cage enclosed within a sound-attenuating box equipped with an exhaust fan, which provided background noise of 60 dB. Box dimensions were 28 x 50 x 20 cm. Prior to testing, animals were handled for 5 min on 4 consecutive days. After this period, they were placed, one at a time, in the testing chamber and left undisturbed for 2 min. CSs were then administered in a manner identical to that used during conditioning, but without the foot shock. The rats' behavior was recorded using a digital video camera and the videos were reviewed to determine the duration of freezing responses, used as an index of fear. The freezing response was expressed as the percentage of time during which there was complete absence of somatic mobility, except for respiratory movements. The assessment of freezing was carried out by one person blinded to the animal's assignment to a specific experimental group.

**Appetitive conditioning.** Rats were placed on a restricted diet to maintain their body weight at approximately 80–90 % of their free-feeding weight. At 1 day before training, rats were given ~1 g of chocolate-flavored sucrose pellets (Bio-Serv, catalog no. F07256) in their home cages to familiarize them with the pellets. For training, rats were placed in the same conditioning chamber employed in the fear conditioning experiment 1 (Rat Test Cage, Coulbourn Instruments,), enclosed in a sound-attenuating box. They were trained to associate the auditory CS (3000 Hz tone, amplified to 75 dB and lasting 8 s) with an appetitive US, namely one chocolate-flavored pellet delivered at the end of the tone into the food cup. The CS–US pairing was presented 28 times with a variable interval during a 60 min session, once per day for 3 consecutive days. In the appetitive conditioned-odor group, almond odors (12 s, 48-s inter-trial interval) were presented using a flow-dilution olfactometer, as in the odor fear conditioning experiment.

*Remote appetitive memory retention* was tested at 1 month after conditioning. Rats were placed in a different apparatus from that used for conditioning, and in a different room, in order to avoid conditioned fear behavior related to contextual cues (Sacco and Sacchetti 2010). The apparatus was the same used for testing fear memories. Animals were placed in the testing chamber for 2 min (pre-CS period) and then exposed to the CS seven times. Retention of memories was measured using the *conditioned discriminative response* tests. *Conditioned discriminative responses* were calculated as the time (in seconds) an animal spent with its head in the food cup, searching for the chocolate-flavored sucrose pellet in response to the CS, according to the formula: (time in food cup during the CS (8 s) and post-CS presentation (17 s) minus the 25 s preceding CS onset.

**Active avoidance training.** Rats were trained in a standard shuttle box (Ugo Basile Shuttle box module). The apparatus was divided into two identical sections by a pair of white opaque Plexiglas dividers. There was also a hurdle on the bottom of the passage that rose 3 cm above the grid floor. Subjects were presented with a 3000 Hz, 70-dB tone CS that lasted 6 s and that coterminated with footshock US (1.2 mA, 2 s). For every training session, the animal was placed in the shuttle box and given 3-min stimulus-free acclimation period. Then, rats received 8 signaled avoidance trials with an average intertrial interval (ITI) of 1 min. Shuttling during the tone led to immediate tone termination and prevented delivery of the footshock US. If no avoidance shuttle was performed during the CS, the US was presented until the rat shuttled (escape response). Training (one session/day) continued for 6 d or until successful avoidance responses were achieved on >80% of the trials. If an animal did not reach criterion by the last session, it was not used in further experiments.

*Memory retention trial* started two weeks after the beginning of the training. Avoidance conditioned response, i.e. shuttling during the tone, was calculated. To get an estimate of baseline activity, the shuttling was also recorded during the acclimation period, before the first CS was presented. A total of three CSs were presented during the retention test.

**Immunohistochemistry**

In both the fear and appetitive memory experiments, at 90 min after completing the memory retention test, rats were deeply anaesthetized and perfused intracardially with 4% paraformaldehyde (PAF). Brains were dissected, stored overnight in the 4% PAF at 4 °C, and then transferred to 0.12 M phosphate-buffer saline (PBS) for 2 days. Brains were sliced coronally (50-μm) on a vibratome and collected in PBS. Free-floating sections were pretreated with 0.3% $H_2O_2$ in PBS to reduce endogenous peroxidase activity. After four rinses, sections were incubated in a blocking solution containing 2% bovine serum albumin (BSA), 2% normal goat serum and 0.2% Triton X-100 for 1 h at room temperature. Sections were then incubated in primary polyclonal rabbit anti-zif268 (1: 5000 dilution, Santa Cruz) antibody in the blocking solution overnight at room temperature. Subsequently, sections were washed with PBS and incubated for 2 h at room temperature with biotinylated goat anti-rabbit antibody (1: 2000 dilution, Jackson Laboratories) followed by 2 h at room temperature in avidin-biotin complex (ABC). Sections were rinsed in PBS. Staining was visualized using the avidin-biotin peroxidase method (Vectastain Elite ABC kit, Vector Laboratories) coupled to diaminobenzidine (0.03%) (DAB, Sigma) as a chromogen. Stained sections were rearranged along the anterior–posterior (AP) direction. Finally, sections were transferred to gelatin-coated slides, dehydrated, and covered with a cover-slip.

**Analysis of immediate early gene expression**

The order of sections was reconfirmed under the microscope and each section was assigned to a corresponding range in the antero-to-posterior (AP) coordinates to the bregma, based on the Paxinos and Watson atlas (Paxinos and Watson 1986). The higher order auditory Te2 cortex was defined on the basis of the Paxinos and Watson atlas, with cortical fields referenced to the Zilles atlas (Zilles 1985). The AP distance between consecutive sections was 50 μm. The analyzed area covered the

whole Te2 along the AP direction from stereotaxic coordinates AP: -6.3 up to -7.8, and was divided into three consecutive regions, with each region extending by 500 µm and corresponding to those described in the Zilles atlas (Zilles 1985). Quantitative analysis of zif268 nuclei was performed using Neurolucida software connected to a microscope via a color CCD camera (Sacco and Sacchetti 2010). Quantification of zif268-positive cells was carried out at X 10 magnification in a blind manner. Immunoreactive nuclei were counted bilaterally using at least three serial sections for each area. The number of nuclei expressing zif268 was quantified in the area of interest. The mean count of each animal was divided by the mean count of the respective naive control group in order to generate a normalized count for each animal. Data were then averaged in order to produce the mean of each experimental group (Sacco and Sacchetti 2010).

**Statistical analysis.** Student's t-test, one-way ANOVA and Newman-Keuls multiple comparisons test were employed for behavioral and immunohistochemical analyses. Levene's test was employed to assess the homogeneity of variance among groups. When the variances among groups were not equal (Levene tesy, $p<0.05$), we used the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test. Pearson correlation was used for zif count and behavioral correlations.

## Results

### zif268 analysis in the Te2 cortex after fear memory retrieval

In order to study Te2 activity related to remote auditory fear memory, rats were trained to associate a tone (CS) with an inescapable foot shock (US) (n = 16). As in our previous study (Sacco and Sacchetti 2010), remote memory retention was assessed 1 month later by measuring freezing behavior in response to the CS in the absence of the US. To isolate cortical activities potentially related to fear memory from activities related to emotional arousal or motor behavior expressed during the memory retention test, we added another group of rats retrieving an olfactory remote fear memory (n = 9), a task that does not involve Te2 participation (Sacco and Sacchetti 2010). Moreover, to avoid any influence of contextual cues, we performed the memory retention test in a completely different apparatus. In the apparatus used for testing memory retention, the freezing response measured during the 2 min preceding CS administration was low and similar between different groups ($F_{(2, 43)} = 0.05$, $p > 0.05$), thus revealing the absence of generalization phenomena (Fanselow 1990; Sacchetti et al. 2002, 2004; Sacco and Sacchetti 2010) (Fig. 1A). Conversely, during CS presentation, the freezing response to the tone (auditory fear-conditioned group) or to the odor (olfactory fear-conditioned group) was higher than in naive animals (n = 21) which had never received the tone previously ($F_{(2, 43)} = 98.7$, $p < 0.001$) (Fig. 1A).

In these experimental groups, we then tracked the expression of zif268 proteins to investigate if certain layer(s) and/or particular regions of the Te2 cortex are specifically engaged by fear memories. For this purpose, we analyzed neuronal activation across the entire Te2 by dividing this cortex into three consecutive regions of around 500µm each, ranging from the AP coordinates relative to the bregma (AP) – 6.3 up to – 7.8 mm (Fig. 1B). In each region, we separately analyzed the activity of the cortical layers (Fig. 1C-D). In each layer, we counted the number of neurons expressing the protein coded by the *zif268* gene in naive rats and in animals conditioned to auditory CSs, as well as in odor-conditioned animals, with the experimenter having no knowledge of the

experimental condition. The mean count of each animal was divided by the mean count of the respective naive control group in order to generate a normalized count for each animal.

*zif268 analysis in the cortical Te2 region extending from - 6.3 to - 6.7 mm from the bregma*

Fig. 2A shows data obtained by counting zif268 protein expression in the -6.3/-6.7 mm range of the Te2 cortex. Auditory fear memory recall resulted in a significant difference of zif268 labeling in layers II/III. In this condition, Levene's test showed that the variances of the data were not equal ($p < 0.05$). The subsequent nonparametric Kruskal-Wallis test revealed significant differences among groups ($p < 0.01$). Mann-Whitney test showed differences between the auditory fear-conditioned group (n. of animals = 21) and the naive (n = 16) and odor-conditioned groups (n = 9) ($p < 0.001$ in both cases), while the latter two groups did not differ from each other ($p > 0.05$). We also detected significant differences in the layer IV. In this layer, Levene's test was not significant ($p > 0.05$). One-way ANOVA showed significant differences among groups ($F_{(2, 43)} = 5.78$, $p < 0.01$). The Newman-Keuls post-hoc test revealed differences between the auditory fear-conditioned group and the naive and odor-conditioned groups ($p < 0.01$ in both cases), while the latter two groups did not differ from each other ($p > 0.05$).

No differences were detected between the groups in layer V ($F_{(2, 43)} = 1.34$, $p > 0.05$) and VI ($F_{(2, 43)} = 1.72$, $p > 0.05$) (Supplementary Fig. 1A).

*zif268 analysis in the cortical Te2 region extending from - 6.8 to - 7.3 mm from the bregma*

In this cortical range, we detected differences in many cortical layers (Fig. 2B). In layers II/III ($F_{(2, 43)} = 15.94$, $p < 0.001$), the "fear-to-tone" group had an increased number of zif268-positive cells with respect to both the naive ($p < 0.001$) and the odor-conditioned group ($p < 0.01$). In layer IV no differences were detected between groups ($F_{(2, 43)} = 1.21$, $p > 0.05$). In layer V ($F_{(2, 43)} = 4.71$, $p < 0.05$) the fear-to-tone group had an increased number of zif268$^{-}$positive cells with respect to naive animals and to the fear-to-odor groups ($p < 0.05$ in both instances). Even in layer VI ($F_{(2, 43)} = 4.02$,

$p < 0.05$) the fear-to-tone group had an increased number of zif268-positive cells with respect to naive animals ($p < 0.05$) but not to the odor-conditioned group ($p > 0.05$) (Supplementary Fig. 1B).

*zif268 analysis in the cortical Te2 region extending from - 7.4 to - 7.8 mm from the bregma*

In this Te2 region, we found statistical differences in many ranges (Fig. 2C). In layers II/III ($F_{(2, 43)} = 32.57$, $p < 0.001$), the fear-to-tone group had an increased number of zif268-positive cells with respect to both the naive ($p < 0.001$) and the odor-conditioned group ($p < 0.001$). Similar results were observed in layer IV ($F_{(2, 43)} = 17.98$, $p < 0.001$), where the fear-to-tone group had an increased number of zif268-positive cells with respect to both the naive ($p < 0.001$) and the odor-conditioned groups $p < 0.001$). In layer V ($F_{(2, 43)} = 5.91$, $p < 0.001$) the fear-to-tone group had a greater number of zif268-positive cells with respect to the naive group ($p < 0.05$) but there was no significant difference between odor- and tone-conditioned rats ($p > 0.05$). In the layer VI, Levene's test showed that the variances of the data were not equal ($p < 0.05$). Kruskal-Wallis test showed significant differences among groups ($p < 0.01$). Mann-Whitney test showed differences between the odor fear-conditioned group and the naive and odor-conditioned groups ($p < 0.01$ in both cases), while the latter two groups did not differ from each other ($p > 0.05$) (Supplementary Fig. 1C).

**Te2 activity after remote appetitive memory recall**

We then addressed the issue of whether and how Te2 activity is modified by an auditory stimulus paired to an appetitive, rewarding US. Rats were trained to associate a pleasant food delivery with an auditory or an olfactory CS. Auditory and olfactory CSs were similar to those employed in the previous fear-conditioned experiments (frequency, intensity, duration). After 1 month, memory retention was assessed by measuring the total time that animals spent searching for the appetitive reward (i.e. food cup entry time; CS – pre-CS in seconds) (Cambiaghi et al. 2015). Memory retention trials were performed in the same apparatus used for testing fear memories. Both the

auditory-(n = 17) and the odor- (n =12) conditioned rats displayed statistically significant conditioned appetitive responses to the CS compared to naive (n = 16) ones ($F_{(2, 42)}$ = 66,65, $p < 0.001$) (Fig. 3A). The zif268 count was then performed in the different regions and layers of the Te2 (Fig. 3B), as for the previous experiments.

*zif268 analysis in the cortical Te2 region extending from - 6.3 to - 6.7 mm from the bregma*

In this range, we observed an increased expression of zif268 proteins in layers II/III ($F_{(2, 42)}$ = 5.42, $p < 0.01$) and layer V ($F_{(2, 42)}$ = 4.43, $p < 0.05$) between rats conditioned to tone CS (n = 17) as respect to rats conditioned to odor CS (n = 12) as well as to naive (n = 16) animals, as shown in Fig. 4A. Conversely, there were no differences between odor conditioned rats and naive animals ($p > 0.05$ in both instances). Layers IV and VI did not show any difference among the three groups ($F_{(2, 42)}$ = 1.41, $F_{(2, 42)}$ = 1.29; $p > 0.05$ in both cases) (Supplementary Fig. 2A).

*zif268 analysis in the cortical Te2 region extending from - 6.8 to - 7.3 mm from the bregma*

In this region we found that tone-conditioned rats had a significant increase of zif268-positive cells with respect to the other two groups in layers II/III ($F_{(2, 42)}$ = 5.22, $p < 0.01$), in layer V (Kruskal-Wallis test, $p < 0.05$) and VI ($F_{(2, 42)}$ = 6.12, $p < 0.01$) (Fig. 4B). In all these layers, no differences were observed between naive and odor conditioned rats ($p > 0.05$ in all instances). Conversely, in the layer IV, tone conditioned animals were different from odor-conditioned rats ($F_{(2, 42)}$ = 3.10, $p < 0.05$) whilst there was no difference between tone-conditioned rats and the naive ones ($p > 0.05$) (Fig. 4B and Supplementary Fig. 2B).

*zif268 analysis in the cortical Te2 region extending from - 7.4 to - 7.8 mm from the bregma*

As shown in Fig. 4C, we observed a significant increase in zif268 expression in the appetitive-conditioned rats compared to the naive group in layers II/III ($F_{(2, 42)}$ = 4.50, $p < 0.05$) but not between tone- and odor-conditioned rats ($p > 0.05$). None of the other layers showed any

differences (layer VI, $F_{(2, 42)} = 1.70$; layer V, $F_{(2, 42)} = 0.41$; layer VI, $F_{(2, 42)} = 0.30$; $p > 0.05$ in all instances) (Supplementary Fig. 2C).

**Te2 activity following fear vs. appetitive remote memory retrieval**

In addition to depicting the neural circuitry that is recruited in the Te2 by fear or appetitive memory retrieval, our data allowed us to compare neuronal activity in the Te2 between the two contrasting emotional memories. To facilitate this comparison, we included data (Fig. 5) that presents the layers and the regions where either the fear- or the appetitive-memory recall induced a statistical significant change of zif268 activity compared to naive groups.

Moreover, in order to investigate whether changes in the activity detected in the different layers correlate with the behavioral responses displayed by rats during fear or appetitive memory tests, we plotted zif268 count versus the individual behavioral responses in both fear (i.e., total freezing time) and appetitive (i.e., total food cup entry time) conditioned rats. In the fear conditioned rats to tone, in the layer II/III, there was no correlation in the 6.3 - 6.7 range (Pearson test, $r = - 0.34$, $p > 0.05$), in the 6.8 - 7.3 range ($r = -0.17$, $p > 0.05$) and in the 7.4 – 7.8 range ($r = 0.46$, $p > 0.05$). Similarly, no differences were detected in all other layers ($p > 0.05$ in all instances). Similar results were obtained in the appetitive conditioned rats ($p > 0.05$ in all cases).

**Te2 activity after the recall of an auditory signaled two-way active avoidance**

Recent findings pointed out that motor activity influences auditory processes in the primary auditory cortex (Remington et al. 2012; Schneider and Mooney 2015; Xiong et al. 2015; Zhou et al. 2014). Thus, the auditory cortex may be part of a sensorimotor system that links auditory stimuli to specific motor responses and, therefore, the differences detected in the present study between fear vs. appetitive paradigms may be due to such the different motor responses paired to tones. Because our recent study showed that Te2 activity in the layer II/III is not related to these motor processes

(Grosso et al. 2015b), it may be that other Te2 layers are recruited by these sensorimotor processes, and specifically the layer V which projects to subcortical structures involved in emotional and motor responses regulation (Paxinos, 2004). To address this issue, we performed zif268 analysis in rats trained to associate an auditory CS to a different motor behaviors, i.e. an active avoidance reaction (Grosso et al. 2015b). In these rats, during memory retention test, the presentation of the CS determined an escape conditioned reaction to the adjacent chamber (n = 6) which is not present in naive rats (n = 8) ($t_{12}$= 0.76; $p < 0.01$) (Fig. 6A).

*zif268 analysis in the cortical Te2 region extending from - 6.3 to - 6.7 mm from the bregma*

Fig. 6B shows data obtained by counting zif268 protein expression in the -6.3/-6.7 mm range of the Te2 cortex. Auditory fear memory recall resulted in a significant difference of zif268 labeling between conditioned and naive rats in layers II/III ($t_{12}$= 2.70; $p < 0.05$) and layer IV ($t_{12}$= 2.59; $p < 0.05$). Remarkably, there were also difference between naive and conditioned rats also in layer V ($t_{12}$= 4.38; $p < 0.005$) and VI ($t_{12}$= 2.59; $p < 0.05$).

*zif268 analysis in the cortical Te2 region extending from - 6.8 to - 7.3 mm from the bregma*

In this cortical range, we detected differences in all cortical layers (Fig. 6C). The “fear-to-tone” group had an increased number of zif268-positive cells with respect to the naive group in layer II/III ($t_{12}$= 2.31; $p < 0.05$); IV ($t_{12}$= 2.34; $p < 0.05$); V ($t_{12}$= 4.53; $p < 0.005$) and VI ($t_{12}$= 2.76; $p < 0.05$).

*zif268 analysis in the cortical Te2 region extending from - 7.4 to - 7.8 mm from the bregma*

In this Te2 region, we found statistical differences in layer II/III ($t_{12}$= 4.91; $p < 0.01$); V ($t_{12}$= 4.22; $p < 0.01$) and VI ($t_{12}$= 4.58; $p < 0.01$). Conversely, there was no difference between conditioned and naive rats in layer IV ($t_{12}$= 0.62; $p > 0.05$) (Fig. 6D).

**Discussion**

In the present study we addressed two related questions. Firstly, we investigated the regions and the layers recruited in the Te2 during the retrieval of remote auditory fear memories. We found that auditory fear memories recruit layers II/III across the entire extension of the Te2. Conversely, layers IV and V were only activated in specific Te2 regions.

Secondly, we investigated how Te2 activity was shaped by presenting identical tones that had been previously paired to appetitive stimuli. Similar to fear memory recall, the retrieval of remote appetitive memories induced a significant increment in zif268-staining in layers II/III. At odds with fear memory recall, however, appetitive memories did not recruit the most posterior region of Te2 cortex.

*Factors determining layer- and regional- differences between groups*

The auditory stimuli employed as CSs were identical in term of frequency, duration, and intensity in both appetitive and fearful memories. Consequently, we can exclude that the observed differences in Te2 activation are due to the processing or memorization of the physical attributes of the auditory CS. In addition, all groups were tested in the same environment, which was completely new and different from the conditioning apparatus. Thus, we can also rule out any influence by contextual cues (Fanselow 1990; Grosso et al. 2015b; Sacchetti et al. 2002, 2004; Sacco and Sacchetti 2010).

Another possible source of differences between appetitive and aversive processes may rely to the different types of neurons recruited by each of these processes. Different types of interneurons and pyramidal cells have been detected in the sensory cortex and may be differentially recruited by emotionally-laden experiences. zif268 expression has been detected in both glutamatergic and GABAergic cells in several brain structures (Ishida et al. 2000). Therefore, further studies are needed to better characterize the neuronal populations engaged in the different layers during fear- and/or appetitive memory processes.

*Layer-specific memory evoked activity in the Te2 cortex*

Layer II/III

In our previous study, we reported that in the Te2 cortex remote fear memory retrieval induced changes in zif268 activity in the ***layers II/III*** at the stereotaxic coordinates around 7.3 mm posterior to the bregma (Sacco and Sacchetti 2010). Here we expanded these findings by showing that layers II/III are recruited across the entire extension of the Te2 cortex and, in addition, we revealed that this phenomenon is neither due to motor behavior nor to fear-evoked arousal. Indeed, when compared with all the other layers, superficial laminae are the most extensively engaged by fear memories.

These data indicate that memory processes engage a largely distributed neuronal population in the layers II/III. At variance with our study, however, Kwon et al. (Kwon et al. 2012) reported that auditory fear memory retrieval did not modify zif268 signals in higher order auditory cortex in mice. The authors suggest that the difference between their study and our previous data (Sacco and Sacchetti 2010) "might result from different behavioral protocols used in each study and probably reflect species differences in functional neural circuit organization in the mouse and rat brains" (Kwon et al. 2012). However, several previous studies have reported memory-related plasticity in laminae II/III in both rats and mice. The disinhibition of selective inter-neurons in the layers II/III is a critical process for learning fear in response to complex auditory CSs (Letzkus et al. 2011). Further studies confirmed the recruitment of layer II/III interneurons in auditory fear memory in mice (Pi et al. 2013; Sarro et al. 2015) Changes in neuronal activity have also been detected in superficial layers during spatial (Maviel et al. 2004) and other hippocampal-dependent (Frankland et al. 2004; Lesburguères et al. 2011) tasks in both rats and mice brain cortices, including the prefrontal, anterior cingulate, parietal and temporal cortices (Frankland and Bontempi 2005). Spine remodeling and growth has been shown to occur in superficial layers during remote memory formation in mice (Lesburguères et al. 2011; Vetere et al. 2011; Xie et al. 2014), and contextual fear

conditioning has been shown to determine a memory-related activation of sparse neurons in layer II of several cortices in mice, which lasts for 2 months (Xie et al. 2014). Taken together, past and the present data support the view that superficial layers represent key sites for memory processes.

Superficial laminae are characterized by integrative processes that may determine the observed intense activation following memory recall. Moreover, in the layers II/III originate many callosal projection to the controlateral auditory cortex (Paxinos 2004). In addition, superficial laminae are intensely connected with many other cortices (Kolb and Tees 1990; Paxinos 2004), like the cingulate, visual and perirhinal cortices (Paxinos 2004) and it has been proposed that such laminar activity reflects the formation of cortico-cortical neural assemblies (Maviel et al. 2004). In line with this idea, our previous study showed that remote fear memories to olfactory, auditory and visual CSs are widely distributed throughout the cortex, with each secondary sensory cortex encoding information about sensory stimuli of a specific modality (Sacco and Sacchetti 2010).

By combining immediate early gene analysis and optogenetic technique, Tonegawa and colleagues identified neurons that are recruited by learning processes and whose activation leads to memory retrieval (Ramirez et al. 2013; Redondo et al. 2014; Tonegawa et al. 2015). These authors propose that "enhanced engram cell-specific synaptic strength is crucial for the retrievability of particular memory engrams, while the memory information content itself is encoded in a pattern of engram cell ensemble connectivity" (Tonegawa et al. 2015). Although these data referred to different brain structures (hippocampus and amygdala) and to memories formed early after training, the enhanced staining of cells we detected in the conditioned rats may be, at least in part, due to the activation of these cell ensemble and related changes in their connectivity.

We also found that layers II/III are widely activated across the entire Te2 following the retrieval of appetitive remote memories, further supporting the involvement of these laminae in memory processes. Given that superficial layers enable cortical-to-cortical connectivity, and previous findings showed that Te1 plays a prominent role in appetitive memories (Xiong et al. 2015; Znamenskiy and Zador 2013), it can be suggested that the activity we observed in these laminae

following appetitive memory recall reflects a functional connectivity between Te2 activity and the Te1 cortex.

The similarity of the results that we observed in layers II/III after both fear and appetitive memory recall raises the question of which information is encoded at this cortical level. Recently, we found that in layer II/III of Te2 following the presentation of two auditory cues previously paired with either pleasant or painful stimuli, a large percentage of cells responds to both experiences but also a small fraction of neurons responds exclusively to one of them. The latter type of neurons signals the valence rather than the salience or the motor responses associated with the stimuli (Grosso et al. 2015b). Accordingly, some cortical interneurons in the auditory cortex were shown to be responsive not only to auditory CSs but also to painful USs (Letzkus et al. 2011; Pi et al. 2013) and appetitive USs (Pi et al. 2013), indicating that the activity of cortical neurons is modulated by emotional information.

Layer IV

At variance with the results obtained in the layers II/III, we found that neuronal activity in *layer IV* changed following fear but not appetitive memory retrieval. In addition, layer IV was significantly engaged by fear memory retrieval only in a subfield of Te2 and never following appetitive memory retrieval. This data suggest that layer IV may not take a key role in emotional memory processes at the level of Te2 cortex or, alternatively, that zif268 staining is not appropriate to reveal memory-related plasticity in this cortical layer.

Layer IV of the Te2 cortex receives axons arriving from several subcortical structures, such as the locus coeruleus, raphe and thalamic nuclei (Arnault and Roger 1990; Kolb and Tees 1990; Paxinos 2004). Concerning thalamic projections, Te2 cortex receives projections from the medial and the dorsal division of the geniculate body (Antunes and Moita 2010; Arnault and Roger 1990; Kolb and Tees 1990). Several studies have indicated that these thalamic nuclei are involved in fear learning processes (Antunes and Moita 2010; Apergis-Schoute et al. 2005; Han et al. 2008; Weinberger

2011) and in remote fear memory storage/retrieval (Kwon et al. 2012). The enhanced zif268 staining detected in layer IV may therefore reflect the activity of such thalamic-to-cortical connectivity during fear memory processes. The fact that zif268 staining in this layer is unchanged in rats retrieving appetitive remote memories suggests that this pathway is not engaged as a result of tones with appetitive-related features. Sensory information about appetitive CSs may reach the Te2 cortex via layers II/III, which is connected to the Te1 auditory cortex (Romanski and LeDoux 1993a) and which also receives some sparse afferents from subcortical nuclei. In addition, a recent study showed that thalamic inputs to the cortex terminate also in the layer V/VI (Constantinople and Bruno 2013), two of the layers in which we found enhanced activity after memory retrieval.

Layer V

Regarding *layer V*, our data depict a circumscribed regional recruitment of this layer by fear memory processes, namely starting from the AP coordinates - 6.8 up to -7.8 mm from the bregma but not in the more anterior part of the Te2. Interestingly, this activity pattern is more circumscribed than that observed in layers II/III, thus indicating a marked difference between these layers. Moreover, in rats trained to actively avoid the CS, layer V activity was enhanced also in the more anterior part of Te2. Active avoidance response is characterized by approaching the adjacent box and it is totally different from the freezing conditioned response, maybe resembling the approach to cup response detected in the appetitive memory processes. In line with this observation, layer V activity was enhanced in the more anterior Te2 region also in appetitive conditioned rats. Therefore, it may be that layer V activity is related to the motor response specifically paired to the auditory CS. Layer V contains neurons that send their axons to subcortical nuclei. The major corticofugal projections are glutaminergic (Paxinos 2004). Unlike the primary auditory cortex Te1, the Te2 sends axons to the lateral amygdala (Romanski and LeDoux 1993a; Shi and Cassell 1997), a key site for fear memory. *In vitro* studies have shown that descending axons from the auditory cortex to the lateral amygdala display long-term potentiation (LTP) (Bissière et al. 2003; Fourcaudot et al.

2009). LTP expression is mediated by a persistent increase in the presynaptic probability of neurotransmitter release at cortical afferents (Bissière et al. 2003; Fourcaudot et al. 2009), that is, synaptic plasticity occurs at the level of cortical descending axon synapses. Intriguingly, Kwon et al. (Kwon et al. 2014) reported that by pairing with footshock the photostimulation of axons in the amygdala that are arising from the secondary auditory cortex and the medial geniculate nucleus was sufficient to elicit during subsequent memory retrieval trial fear-related responses. In accord with these findings, we found that fear memory retrieval correlates with Te2-to basolateral amygdala connectivity (Cambiaghi et al. 2016). This data may explain why in the present study we did not detect any correlations between Te2 activity and behavioral conditioned responses. More likely, emotional behavior towards learned auditory stimuli results from an integrate brain network made, among others, by the Te2 and the amygdala.

Layer V neurons may also send information to other structures involved in learned fear. For instance, auditory cortical outputs by passing through a cortico-pontine-cerebellar pathway arrive at the cerebellar vermis (Azizi et al. 1985), another brain structure that is involved in fear learning (Ruediger et al. 2011; Sacchetti et al. 2002, 2004, 2009).

Layer VI

Finally, our data have shown that fear memory retrieval also enhanced zif268 expression in *layer VI*. Most terminal boutons arising from the auditory cortex are small and end in small caliber dendrites in the medial geniculate body (Barlett et al. 2000; Paxinos, 2004). Most probably these boutons arise from the pyramidal cells of layer VI (Paxinos, 2004). Given that we also observed a similar result in animals retrieving olfactory fear memory, it is difficult to distinguish whether the activity of this layer reflects memory or other factors, such as an enhanced fear state and/or the freezing response.

Collectively, our findings provide new information on the neural circuitry within the auditory cortex that processes and stores fear and appetitive auditory memories. These data suggest that the

superficial layers, traditionally thought to perform associative processes and enable cortico-cortical connections, are broadly recruited by both appetitive and fear memories, i.e. they may represent the laminae where associative memory information are processed and encoded. Conversely, the activity of the layers which provide afferents to the cortex from subcortical nuclei, namely layer IV, or which send efferents to subcortical nuclei, i.e., layer V, is determined by the affective/motivational and charges of sounds as well as by the associated motor behavior, possibly reflecting the existence of different neural circuits to and from the auditory cortex in appetitive and fear memories.

*Te2 participation in emotional remote memory*

On a more general level, our results provide information on the possible role(s) covered by Te2 in remote emotional memory processes. The differences observed between groups which perceived identical tones indicate that Te2 activity does not merely reflect auditory stimuli processing. Instead, the emotional-motivational charge associated with sound, acquired during emotionally-laden experiences, strongly wires the layers and regions of Te2. This concept is in line with previous results showing that the Te2 is not significantly activated by non-associative auditory processes, such as long-term habituation to neutral sounds (Gonzalez-Lima and Scheich 1985; Gonzales-Lima et al. 1989) and that lesions in the higher order sensory cortices did not destroy the long-term ability to recognize the physical features of the perceived stimuli (Sacco and Sacchetti 2010). Accordingly, novel and familiar sounds activate Te2 neurons in a similar manner in the absence of conditioning (Sacco and Sacchetti 2010; Wan et al. 2001), while neural activity in this area increases significantly if the sounds had previously acquired a behavioral value (Cambiaghi et al. 2016; Carretta et al. 1999; Kwon et al. 2012; Grosso et al. 2015b; Sacco and Sacchetti 2010). Similar results were reported by Bao et al. (Bao et al. 2001), who showed that, after pairing of auditory stimuli presentation to ventral tegmental area stimulation, “strong, sharply tuned responses to the paired tones also emerge in a second cortical area [Te2], whereas the same stimuli only evoke

poor or non-selective responses in this second cortical field in naive animals." Furthermore, beside Te2, several recent studies showed that also the neurons in the primary auditory cortex Te1 undergo profound and selective changes during either appetitive and aversive learning tasks (David et al. 2012; Fritz et al. 2005a; Yin et al. 2014). Remarkably, the valence of these changes was strictly linked to the task reward structure, rather than to peripheral motor responses (David et al. 2012; Fritz et al. 2005a; Yin et al. 2014), in line with our present data. Thus, the present study, together with past research, supports the view that the activity of the auditory cortex is profoundly influenced by the emotional information assigned to sensory stimuli and raises the intriguing possibility that learned emotional value is a property of stimulus representation in the sensory cortex.

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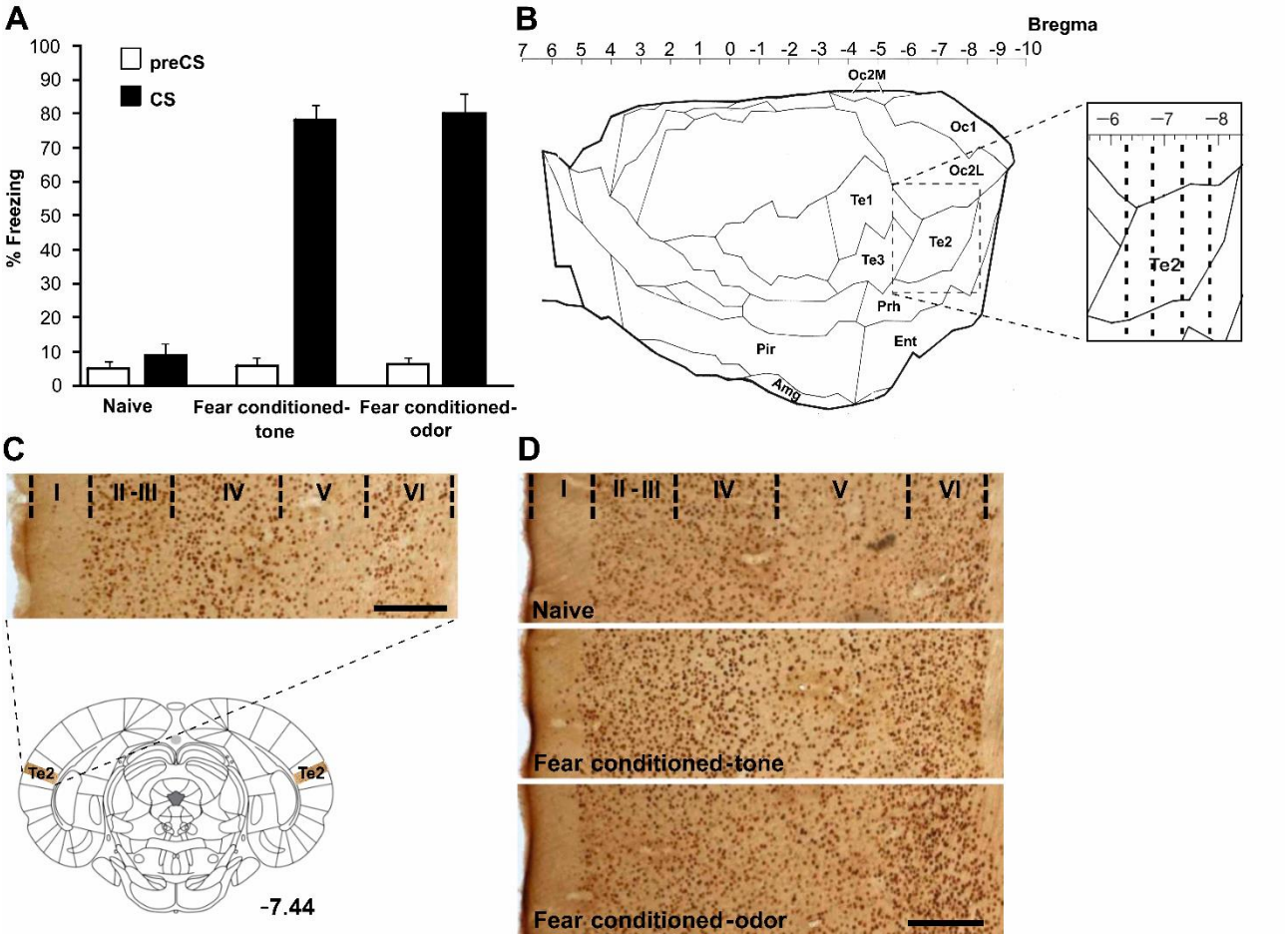
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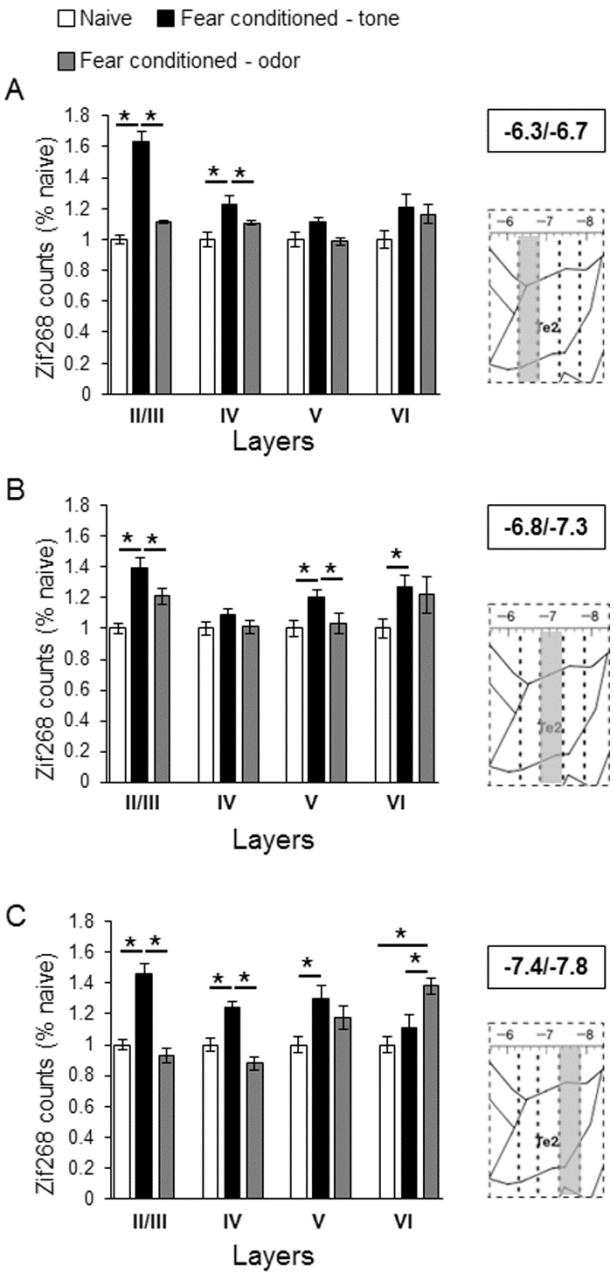
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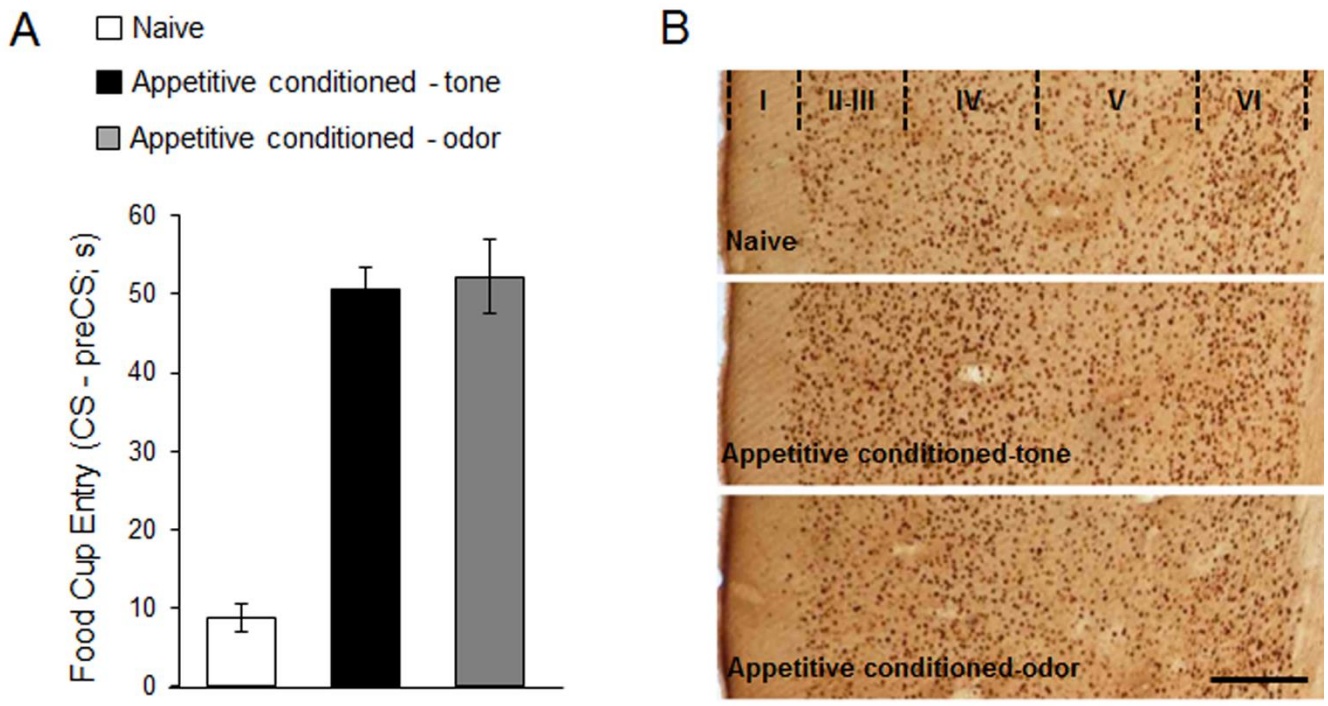
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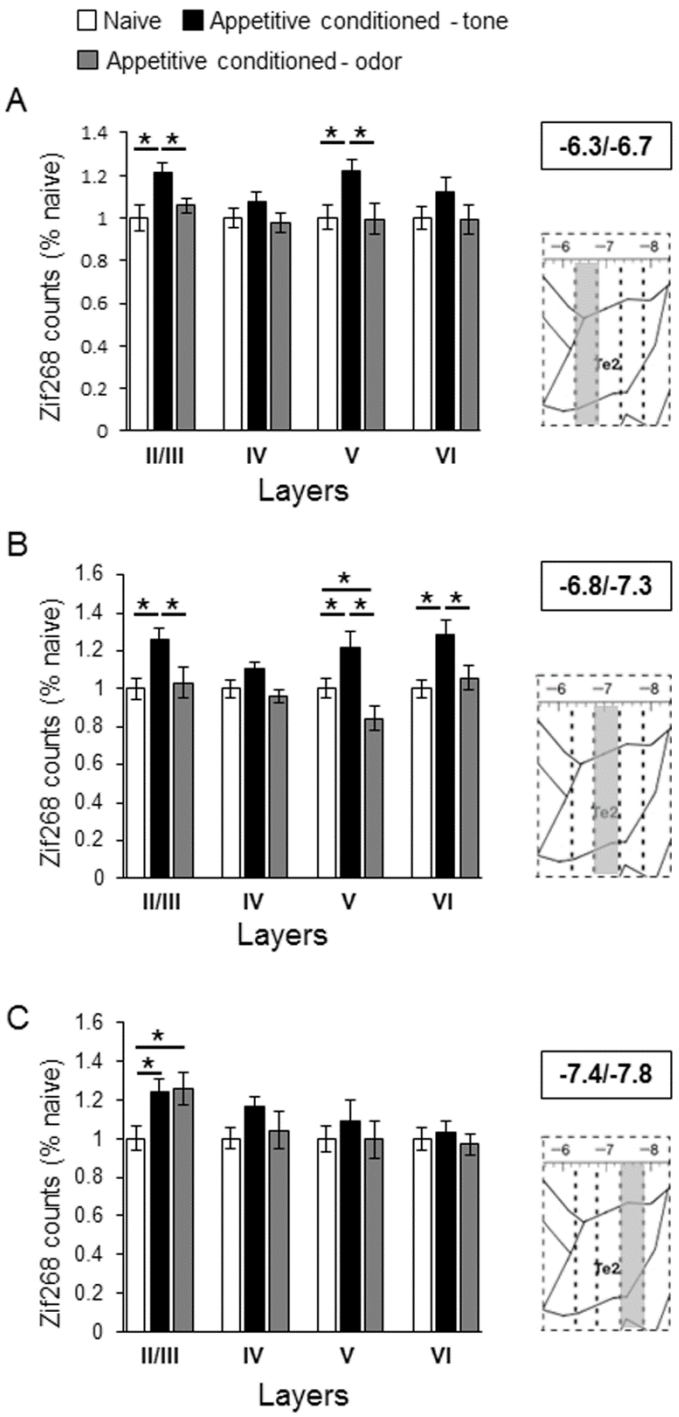
Naive
Appetitive conditioned - tone
Appetitive conditioned - odor
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Zif268 counts (% naive)
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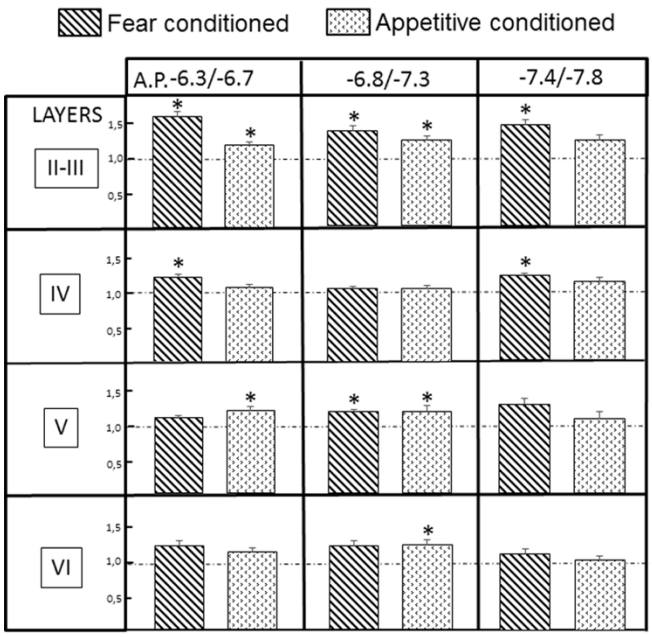
Te2


Fear conditioned
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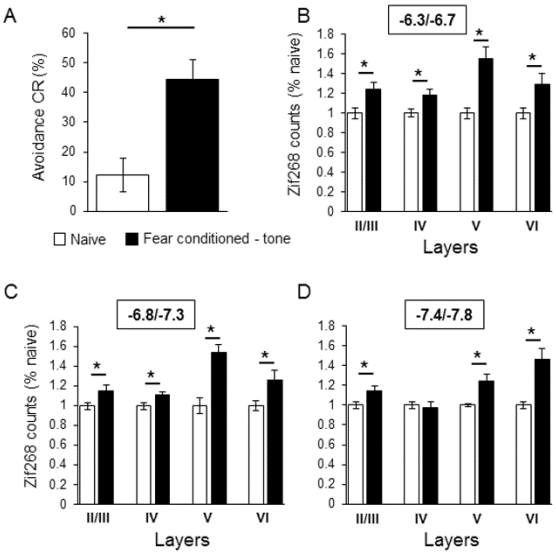
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Fear conditioned - tone
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Zif268 counts (% naive)
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-7.4/-7.8

**Captions to figures**

**Figure 1. Remote fear memories and the Te2 cortex.** (*A*) Long-term memory retention was evaluated at 30 days after conditioning by measuring freezing at 2 min before (pre-CS, empty columns) and during CS presentation (CS, black columns) in rats conditioned to a tone (n = 16), an odor (n = 9) or after the presentation of a similar tone in naive (n = 21) rats. All data are presented as means ± SEM. (*B*) Te2 cortex was divided into four consecutive regions along the anterior-posterior (AP) direction from - 6.3 to – 7.8 mm from the bregma. Plates were adapted from Zilles (1985). Amg: Amygdaloid body; Ent: Entorhinal area; Oc1: Occipital cortex, area 1; Oc2L: Occipital cortex, area 1 lateral part; Oc2M: Occipital cortex, area 1 monocular part; Pir: Primary olfactory cortex; PRh: Perirhinal area; Te1, Te2 and Te3: Temporal cortex area 1, 2 and 3. (*C-D*) Representative images of zif268 protein expression in the layer I-VI of Te2 taken at the AP coordinate of 7.44 mm from the bregma in the naive, fear conditioned-tone and fear conditioned-odor groups. Scale bars, 200 μm

**Figure 2. Layer and region-specific Te2 activity following auditory fear memory retrieval.** (*A*) (Left) Histogram showing zif268 counts normalized on naive group (% of naive) in layers II/III, layer IV, layer V and layer VI in naive (n. of rats = 21), fear-conditioned-tone (n = 16) and fear-conditioned to odor (n = 9) rats. (Right) Schematic illustrating Te2 region extending from AP coordinates: -6.3 to -6.7 mm from the bregma. In (*B*) the Te2 AP -6.8/-7.3 region (right in grey) is shown, along with the zif268 expression analysis (left). (*C*) Schematic exemplifying the Te2 AP -7.4/-7.8 region (right in grey) and the zif268 count (left). * $P < 0.05$. Data shown are mean ± SEM.

**Figure 3. Appetitive remote memories and Te2 activity.** (*A*) Remote appetitive memories were tested 30 days after conditioning by measuring the total time animals spent searching for an award (i.e. food cup entry time; CS – preCS in seconds) for auditory-conditioned animals (n. of rats = 17, black columns), odor conditioned rats (n = 12) or after the presentation of a similar tone in naive rats (n= 16, empty columns). (*B*) Representative images of zif268 protein expression in layers I-VI of the Te2 cortex, taken at the AP coordinate of 7.44 mm from the bregma. Scale bars, 200 μm. Data shown are means ±SEM.

**Figure 4. Layer and region-specific Te2's activity following auditory appetitive memory retrieval.** (*A*) (Left) Histogram showing zif268 counts normalized on naive group (% of naive) in layers II/III, layer IV, layer V and layer VI in naive (n = 16), tone- (n = 17) and odor- (n = 12) appetitive-conditioned rats. (Right) Schematic illustrating the Te2 AP -6.3/-6.7 region analyzed for zif268 expression. In (*B*), the schematic representation of the Te2 AP -6.8/-7.3 region (right in grey) is illustrated, along with the zif268 expression analysis (left). (*C*) zif268 expression count (left) and schematic illustrating the Te2 AP -7.4/-7.8 region. * $P < 0.05$. All data are means ± SEM.

**Figure 5. Schematic figure showing significant changes in the fear and appetitive conditioned animals as regard to naive and odor-conditioned groups.** In the Figure, we reported the data of auditory fear and appetitive conditioned rats which were statistically different from both naive and odor-conditioned groups. The data refereed to zif268 count normalized over naive for both fear and appetitive conditioned rats in different antero-posterior ranges (A.P.; in mm from bregma) in different layers (II/III to VI). We marked with * the data which were significantly different from both the naive and the odor conditioned groups ($P < 0.05$). All data are mean ± SEM.

**Figure 6. Te2 activity following the active avoidance learning paradigm.** *(A)* Percentage of active avoidance conditioned responses (CR) (CS-preCS shuttle response) displayed by conditioned

rats (n. = 6) and by naive (n= 8) animals. *(B)* (Left) Histogram showed zif268 counts normalized on naive group (% of naive) in layers II/III, layer IV, layer V and layer VI in naive and conditioned rats. (Right) zif268 analysis performed in the Te2 region extending from AP coordinates: -6.3 to -6.7 mm from the bregma. In (*C*) zif268 expression analysis in the Te2 AP -6.8/-7.3 region. (*D*) zif268 count in the Te2 AP -7.4/-7.8 region. * $P < 0.05$. Data shown are mean ± SEM.